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Comparative study of the quantification of thin-layer chromatograms of a model dye using three types of commercial densitometers and image analysis with ImageJ

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ABSTRACT

A comparison of slit scanning densitometry, videodensitometry, flatbed scanner densitometry, and image analysis is presented for quantification of high performance thin layer chromatograms containing zones of the model dye rhodamine B. For the first three methods commercial instruments were used, i.e, CAMAG TLC Scanner 3, CAMAG Videodensitometer, and AR2i ChromImage flatbed scanner, respectively. Image analysis was performed using a digital camera and free ImageJ software. Information is provided on the construction and applications of the commercial instruments, and a review of recent publications on thin layer chromatography with image analysis is provided.

KEYWORDS: thin layer chromatography, planar chromatography, TLC, densitometry, videodensitometry, image analysis, ChromImage, ImageJ

1. INTRODUCTION

The steps of modern quantitative thin-layer chromatography (TLC) and high performance TLC (HPTLC) were reviewed in a previous article in this journal [1], including sample preparation, application of standard and sample zones, mobile phases, plate development, zone detection, zone identification and confirmation, documentation of results, and densitometric chromatogram evaluation. This paper discusses the four current, different types of densitometry and presents comparative experimental data using them for the quantification of a model dye. For each type of densitometry, a series of standard zones were scanned to measure their absorption of radiation, and a calibration curve was generated from the peak areas versus corresponding weights. This calibration curve was then used for the interpolation of the weights of bracketed unknown sample zones based on their areas.

2. Commercial densitometers

A paper on the chronology of TLC focusing on instrumental progress included dates of the development of different commercial brands and types of densitometers, which began in the 1960s [2]. Photographs and descriptions of the following densitometers in use today were presented in a series of reports on analytical instrumentation: CAMAG TLC Scanner 3 and CAMAG Videodensitometer with a 3-CCD camera [3]; J&M TIDAS TLC 2010 fiber optic TLC scanner with diode array detector (DAD), CAMAG Videodensitometer with a digital camera in place of the CCD camera mentioned above, and AR2i ChromImage flatbed scanner densitometer [4]; and Desaga CD 60 densitometer [5]. The Scanner 3 has been replaced by the CAMAG Scanner 4 that has essentially the same light path arrangement [3] but a smaller footprint, extended scanning range (190-900 nm versus 190-800 nm), increased signal to noise ratio, a redesigned scanning stage that improves plate loading and handling, and more reliable electronics resulting in better reproducibility according to CAMAG. The Scanner 3

is currently the most widely used densitometer worldwide as reported in the TLC literature, with hundreds of yearly references documented by Sherma in biennial reviews of planar chromatography published since 1970 [6]; this instrument, the Desaga CD 60, and the DAD scanner are classified as slit scanning densitometers. Advantages and applications of TLC with DAD scanning were described in detail by Tuzimski in a recent book chapter [7]. Applications reported for the Videodensitometer and ChromImage are given in the biennial reviews [6] along with those for the slit scanning densitometers.

3. Image analysis

The latest trend in densitometric chromatogram quantification is the use of so called "image analysis". The Videodensitometer and ChromImage mentioned above technically are based on image analysis, but today the term usually refers to the use of a digital camera (phone, compact, or professional) or office type scanner to obtain images of the chromatograms on a plate, uploading on a computer, and qualitative and quantitative evaluation using various available software programs without the need to purchase an integrated commercial instrument.

There are several options for image analysis software. One is ImageJ, a free software that can be downloaded for both PC and Mac from the U.S. National Institutes of Health (NIH) website [8]. Use of the MATLAB program with Imaging Processing Toolbox extension has also been reported [9]; this program is available for a 30 day free trial, and, depending on the version of the program, it can cost from $ 50 to $ 2,150. Another software is Sorbfil TLC Videodensitometer from Sorbpolymer, Russia, which is also available for a 30 day free trial, after which it costs € 450 (~$ 620) [10].

From a personal communication [11], we learned about two other software programs for image analysis. ImageDecipher-TLC (BioDit Technology, Co. [12]) software supports images only in bmp format and quantifies compounds based on zone area only manually. JustTLC [13] performs quantitative analysis in jpg format based on zone volume by measuring the zone area and color intensity, but it can only operate by automatically converting all images into grayscale. Sorbfil supports images in jpg and bmp format and operates similarly to Image Decipher-TLC, the difference being it does not allow inversion of the images and that detection and quantification based on zone areas can be made only automatically.

An increasing number of applications are being reported each year for the TLC/HPTLC analysis of a variety of analytes in different sample matrices using image analysis. The remainder of this section gives a selective review of applications of this relatively new densitometric approach.

Olech *et al.* studied the antiradical activity of plant material on reversed phase (RP) C18 (octyldecylsilyl) bonded silica gel plates developed with methanol-water-*o*-phosphoric acid (45:54:1); antiradical activity of zones was detected using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical reagent. Specific instructions on taking photographs of chromatograms and use of ImageJ were reported in this paper [14]. ImageJ was also used with a flatbed scanner to analyze free radical scavenging activities of polyphenolic compounds isolated from *Medicago sativa* and *Medicago truncatula* aerial parts on silica gel $60F_{254}$ aluminum plates developed in acetonitrile-water-chloroform-formic acid (60:15:10:5) mobile phase [15]. Again with an office flatbed scanner, ImageJ was used in the determination of laxative rhein content in *Casssia fistula* pod extract on silica gel $60F_{254}$ aluminum plates with ethyl acetate-methanol-water (100:17:10) mobile phase; the colored zones absorbed maximally at 435 nm [16]. ImageJ was used for quantification of ochratoxin A in red wine [limit of detection (LOD) and limit of quantification (LOQ) 0.1 μg/L] using a CCD camera for recording intensities of the naturally fluorescent analyte zone images on an HPTLC silica gel 60 layer illuminated with a UV lamp [17].

Sorbfil TLC Videodensitometer software and an office scanner were used to quantify various diterpenoids in *Andrographis paniculata* by silica gel TLC with dichloromethane-toluene-ethanol (6.5:2.5:1.5) mobile phase and *p*-anisaldehyde-sulfuric acid detection reagent [18]. Furthermore, a digital camera and Sorbfil were used to quantify paracetamol and caffeine from pharmaceutical preparations on HPTLC $C18WF_{254}$ plates with

methanol-glacial acetic acid-water (25:4.3:70.7) mobile phase; the chromatograms containing the fluorescence quenched zones on the F-plate containing a fluorescent phosphor were photographed in a UV chamber [19]. Ceftriaxone sodium was determined in pharmaceutical dosage forms on $C18F_{254}$ plates developed with 15% (w/v) ammonium acetate pH 6.2 buffer-methanol-acetonitrile (12:0.5:0.25) using photography with a digital camera inside a UV cabinet and Sorbfil [20]. Sibutramine was determined in adulterated herbal slimming formulations using silica gel $60F_{254}$ plates developed with toluene-hexane-diethylamine and Sorbfil after digital scanning of the plate [21].

TLSee [22] with image capture by an office scanner quantified disulfiram on $C18F_{254}$ aluminum plates developed with methanol-water (9:1) and detected with cupric sulfate reagent [23]. ImageQuant TL v. 2003 [24] image analysis software was used in the silica gel TLC quantification of steroid drug intermediates formed during bioconversion of soysterols using a silica gel 60 plate, benzene-ethyl acetate (5:1) mobile phase, and ceric ammonium sulfate-sulfuric acid detection reagent; the colored zones were scanned with a Laser Jet office scanner and quantified with the Image Quant, which works with the digital image in grayscale from the scanner, enables selection of the desired zone, and determines the area and intensity of the standard versus sample zones [25]. Curcuminoids that were methanol-extracted from *Curcuma longa* (turmeric) were quantified on a silica gel 60_{254} aluminum plate using chloroform-hexane-methanol (1:1:0.1) mobile phase to yield a chromatogram with three naturally colored yellow zones that was captured by an HP Scan Jet 3500C digital scanner followed by quantification of each using Adobe Systems Photoshop 7.0 software [26, 27]. MATLAB with Imaging Processing Toolbox was applied to quantify the colored image of a developed cellulose TLC plate with Co, Cr, and Cu metal ions as models [28]. Threanine in tea extracts was determined on silica gel $60F_{254}$ plates developed with n-butanol-acetone-acetic acid-water (7:7:2:4); quantification involved taking photographs of the plates with a Fujifilm digital camera and then analyzing the green channels of the photographs by free CP Atlas 2.0 software [29] using the "dark on light" option [30].

Sima *et al.* [31] developed a method for simultaneous analysis of two catechol related compounds, carbidopa and levodopa, on $C18WF_{254}$ plates with pH 3 citrate buffer-methanol-formic acid (96:4:5) mobile phase and DPPH free radical detection reagent; chromatogram image acquisition was with a BioDit TLC Scanner, and ImageDecipher-TLC, Sorbfil TLC Videodensitometer, and Just TLC were used for digital image processing and quantification of the compounds on the plate. In all cases, the area and volume of the chromatographic zones were proportional to the amount of compound applied on the TLC plate.

Abou-Donia *et al.* [32] reported a standardized method for assessment of acetylcholinesterase inhibitory activity of different plant extracts using silica gel HPTLC with chloroform-methanol (85:15) mobile phase and an *in situ* autobiographic method with which active zones showed up as white (bleached) against a yellow background. Captured digital camera jpg images were enhanced and unified with Adobe Photoshop 7.0 program, and three image analysis software packages were compared: ImageJ, JustTLC, and SorbfilTLC. ImageJ proved to be best based on sensitivity, linearity, and precision.

4. EXPERIMENTAL

Comparison of three commercial densitometers, i.e., Scanner 3, Videodensitometer, and ChromImage, with digital camera/ImageJ image analysis for the quantitative HPTLC determination of rhodamine B from a four-dye standard mixture was made based on accuracy (recovery), precision, linearity, and sensitivity (LOQ) as described previously [33].

4.1. HPTLC

HPTLC was performed on 20 cm x 10 cm silica gel $60F_{254}$ GLP plates (EMD Millipore Corp., Billerica, MA, an affiliate of Merck KGaA, Darmstadt, Germany; #5642-6) after they were prewashed by development in dichloromethane-methanol (1:1) to remove any impurities. Test dye mixture 1 (Analtech, Newark, DE, USA, Catalog 30-01) containing 1.00 mg mL^{-1} each of Fast Green FCF (aqua blue), rhodamine B (red), Bismark brown Y (yellow), and Sudan 4 (violet-pink) was diluted to 0.045 mg mL^{-1} (standard S1)

and 0.060 mg mL^{-1} (standard S2) with methanol. Five different volumes of S1 were applied (2.00, 4.00, 6.00, 8.00, and 10.0 µL) in order to produce calibration curves of peak area versus corresponding weight, while 4.00 µL of S2 was applied in triplicate as "known unknown" samples.

Several dilutions were made in order to determine the LOQ of all four densitometry methods. LOQ was defined as the weight of the lowest standard in the calibration curve. It was determined experimentally that a 1/50 dilution of S1 and S2 can be successfully scanned with the Scanner 3, while the other three instruments allowed a dilution of only 1/5 for both S1 and S2 standards. In operation of the Scanner 3 the light beam needs to be manually directed on the lowest standard initial zone, and that was not visible below 1/50 dilution. For the other three methods after capturing the image either with the 3-CCD camera, flatbed scanner, or smartphone camera, the image of lowest standard zone needs to be visible for proper quantification. Lower dilutions than 1/5 of S1 and S2 could not been seen once the image was captured, and, therefore, were not available for quantification using the computer software. For Scanner 3 analysis, this led to a final concentration of 9.0 x 10^{-4} and 1.2 x 10^{-3} mg mL^{-1} concentrations for S1 and S2, respectively, and for analysis by other three instruments the final concentrations of S1 and S2 were 9.0 x 10^{-3} and 1.2 x 10^{-2} mg mL^{-1}, respectively. Therefore, LOQ for the Scanner 3 was 1.8 ng, which was 10 times lower than for the other three methods.

In order to determine the maximum linear range of the S1 standard, an extended calibration curve was also constructed to examine the peak areas versus the corresponding spotted weights. For the Scanner 3 calibration curve S1 had a 9.0 x 10^{-4} mg mL^{-1} concentration, while for the other methods S1 was 9.0 x 10^{-3} mg mL^{-1}. Six different volumes of S1 were spotted (2.00, 4.00, 6.00, 8.00, 16.0, and 32.0 µL) in order to produce the extended calibration curve.

S1 and S2 were applied to plates by use of a CAMAG Linomet IV automated spray-on band applicator fitted with a 100 µL syringe and operated with the following settings: 6 mm band length, 4 s μL^{-1} application rate, 10 mm s^{-1} table speed, 4 mm distance between bands, 1.0 cm distance from the bottom of the plate, and spotting tracks 2-9. The plates were developed to 8 cm beyond the origin with ethyl acetate-methanol-water (80:20:20) in the front trough of a CAMAG HPTLC twin-through chamber presaturated with the mobile phase vapors for 15 minutes; an Analtech saturation pad was placed in the mobile phase contained in the back trough. After development, the plates were dried in a fume hood for approximately 15 min, and the rhodamine B was quantified with the four different types of densitometry.

4.2. Densitometry

In order to obtain the calibration curve in each of the four analyses, the weights of the S1 zones were correlated to their scan areas. The weights of the bracketed S2 zones were interpolated from the calibration curve based on their areas. The assay value was calculated for each sample analysis using the following equation: % = (interpolated experimental weight/applied theoretical weight) x 100.

4.2.1. Scanner 3

The areas of the S1 and S2 zones were measured immediately after plate development in the absorbance-reflectance mode with the halogen-tungsten visible light source set at 547 nm (wavelength of maximum absorption of rhodamine B on the plate as determined previously by measurement in the spectral scanning mode of the instrument). The operation parameters 4.00 x 0.45 Micro slit dimensions and scanning rate of 20 mm s^{-1} were used. The winCATS software automatically creates calibration curves by both linear and the second order polynomial regression and gives the interpolated weights of samples directly.

4.2.2. Videoscanner

The instrument consists of a Reprostar 3 lighting system and 3-CCD camera with zoom objective. The VideoStore 2 software was used to photograph the chromatograms under visible light with a camera aperture setting of 11. In order to perform the densitometric analysis, VideoScan software was used to scan the sample and standard zones and to produce linear and the second-degree polynomial calibration curves relating standard zone weights to their respective areas. The settings

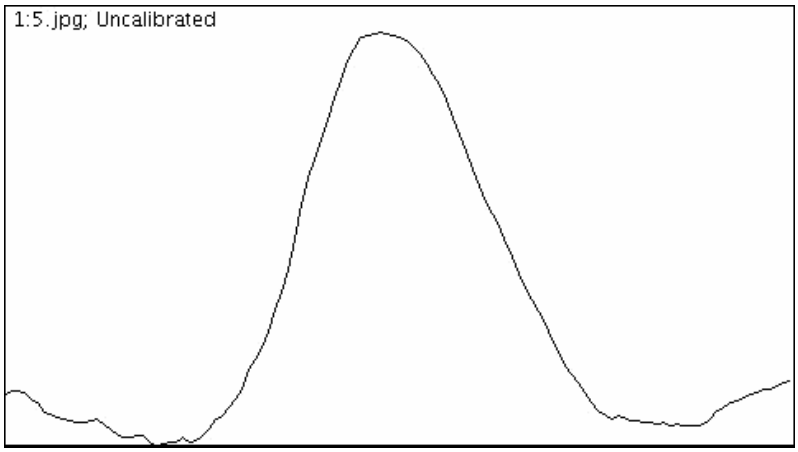

Figure 1. An example of a densitogram peak after denioising and baseline drift removal in ImageJ. The units are y-axis = signal and the x-axis = R_F value.

of the VideoScan software were as follows: 5 pixels as the minimum peak width, 100 pixels minimum peak height, 300 pixels minimum peak area, and 5 filter width; the weights were calculated automatically by the VideoScan interpolation. However, VideoScan did not provide the calibration curve R-values as does the Scanner 3; these were found by creating the same calibration curves in MS Excel.

4.2.3. ChromImage

The procedure for scanning a plate and evaluation of the image with the Galaxie software described by Halkina *et al.* [33] was closely followed. The knob at the back of the scanner was switched to "VIS" for the visible light mode. Galaxie software was used for the acquisition of the chromatogram image, for calibration curve production and the interpolation of the sample weights by using both linear and polynomial regression.

4.2.4. ImageJ

The procedures for taking a photograph of a developed plate with a digital camera and subsequent image analysis as described by Olech *et al.* [14] were followed with certain adjustments. Since a 1080p HD/Google Nexus 4 smartphone camera was used, there was no tripod, but the camera was set in a consistent position in order to minimize any distortions and disturbances; all photographs were taken under the same daylight conditions. In order to remove noise the median filter was used, and the high-pass 2D filter implemented in ImageJ was used to remove the baseline drift caused by non-homogenous illumination. The peaks (Figure 1) were then processed as described [14]. Because ImageJ does not allow direct production of calibration curves as with the three commercial densitometers [34], the calibration curve was constructed and sample weights interpolated in MS Excel.

5. RESULTS

Table 1 shows the results obtained from all four instruments. The calibration plot for S1 was 0.999 for every technique using polynomial regression, and 0.999 for every technique using linear regression except for the ChromImage. S2 assay values ranged from 96-102% and relative standard deviation (RSD) 0.1 to 1% (n = 3), therefore accuracy and precision were excellent in all cases.

Table 2 shows the results for the extended calibration curves for all four instruments, with polynomial and linear regression. Only polynomial calibration curves created from the Scanner 3 and Videoscanner had 0.999 R^2 values.

6. DISCUSSION

Our data offers insight into some of the advantages and disadvantages of the densitometry methods studied. The Scanner 3 gave an LOQ 10 times lower than that found for other three instruments. This is the only instrument that

Table 1. Comparative experimental data on the determination of rhodamine B using the four methods.

Densitometry technique	Calibration curve regression	R^2	Theoretical weight (pg)	Sample weight (pg)	Sample assay (%)	Standard deviation	RSD (%)
Scanner 3: 1/50 dilution	Linear	0.999	96.0	95.8	100	0.4	0.4
	Polynomial	0.999		97.8	102	0.4	0.4
Videodensitometer: 1/5 dilution	Linear	0.999	960	966	101	10	1
	Polynomial	0.999		966	101	10	1
ChromImage: 1/5 dilution	Linear	0.996	960	963	96	1	0.1
	Polynomial	0.999		955	99	1	0.1
ImageJ: 1/5 dilution	Linear	0.999	960	951	99	2	0.2
	Polynomial	0.999		937	98	2	0.2

Table 2. Extended calibration curves for the determination of rhodamine B using the four methods.

Instrument	R^2 calibration curve	
	Linear	Polynomial
Scanner 3	0.987	0.999
Videodensitomer	0.997	0.999
ChromImage	0.987	0.989
ImageJ	0.973	0.982

allowed selection of the optimal measurement wavelength for the analyte by setting the grating monochromator, which can result in greater analytical sensitivity and selectively. The winCATS software is compliance ready for GLP (good laboratory practice)/GMP (good manufacturing practice) and 21 CFR (Code of Federal Regulations, U.S. Food and Drug Administration) guidelines, ensuring data integrity and tracking.

The Videodensitometer has visible and short and long wavelength UV light sources, but particular wavelengths within these ranges cannot be selected. It is noteworthy that this instrument is convenient for taking photographs of plates for presentations and publications of research work, which is also true for the ChromImage and camera used with ImageJ. These three methods do not require immediate scanning of a developed plate having zones that decompose quickly (e.g., carotenoid pigments), as does the Scanner 3, because chromatogram images captured immediately can be processed for quantitative data with the software at any later time. The ChromImage has visible and short wavelength UV sources, and its Galaxie software was the most difficult to use (analysis of each lane had to be completed individually; it was not possible to analyze the whole plate at the same time).

ImageJ allows rapid, convenient, and inexpensive chromatogram quantification with an available camera and free software. In our study with the model dye, it yielded results that were comparable to the three commercial instruments except for the higher LOQ compared to the Scanner 3. The Videodensitometer, ChromImage, and ImageJ used three different types of image capture (i.e., CCD camera, scanner, and smartphone digital camera, respectively) and three different softwares for image analysis, but all gave the same LOQ.

In future research we will apply image analysis to the determination of a light sensitive analyte in order to evaluate the effect of reduced decomposition of zones on the plates. We will compare different cameras and scanners for optimum capture of chromatogram images, and we will compare ImageJ to other image analysis software available to us, such as those mentioned in Section 3, for factors including sensitivity, linearity, accuracy, precision, and ability to directly give linear and polynomial regression calibration

curves with their R-values and interpolate unknown sample weights, contrary to ImageJ. We will compare results for compounds with different visible colors, and we will study different approaches for obtaining the best chromatogram images by photographing under 254 and 366 nm UV radiation in order to quantify compounds that quench fluorescence on F-plates and naturally fluorescing compounds on plates without a fluorescent indicator, respectively.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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